PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

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03.DEC.2001*067896

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NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	29.11.2001
Applicant's or agent's file reference	IMPORTANT NOTIFICATION
LPB/P32058WO	

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/03543	15/09/2000	17/09/1999

Applicant
UNIVERSITY OF LEEDS et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Cleere, C Tel.+49 89 2399-7713

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
LPB/P32058WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/03543	15/09/2000	17/09/1999
International Patent Classification (IPC) or national classification and IPC C12N15/00		
Applicant UNIVERSITY OF LEEDS et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09/04/2001	29.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Trommsdorff, M Telephone No. +49 89 2399 7361

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03543

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-3,5-17	as originally filed			
4	as received on	03/10/2001	with letter of	03/10/2001

Claims, No.:

1-21	as received on	03/10/2001	with letter of	03/10/2001

Drawings, sheets:

1/4-4/4	as originally filed

Sequence listing part of the description, pages:

1-3, filed with the letter of 04.12.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☒ furnished subsequently to this Authority in written form.

   ☒ furnished subsequently to this Authority in computer readable form.

   ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-13, 15-21
	No:	Claims	14
Inventive step (IS)	Yes:	Claims	1-13, 15-21
	No:	Claims	14
Industrial applicability (IA)	Yes:	Claims	1-21
	No:	Claims	

2. Citations and explanations
   see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

1. Cited documents

The following documents (D) are referred to in this communication; the numbering is the same as in the search report and will be adhered to in the rest of the procedure:

D1: PEREDELCHUK M Y ET AL: 'A method for construction of E. coli strains with multiple DNA insertions in the chromosome' GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, GB, ELSEVIER SCIENCE PUBLISHERS, BARKING, vol. 187, no. 2, 18 March 1997 (1997-03-18), p. 231-8, ISSN: 0378-1119

D4: ZUBKO ELENA ET AL: 'Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes.' NATURE BIOTECHNOLOGY, vol. 18, no. 4, April 2000 (2000-04), p.442-5, ISSN: 1087-0156

2. Re Item II
Priority

2.1. The priority has been checked: the descriptions of the priority document and of the application as filed appear to have the same content. Thus, the priority is valid and it is assumed that all the claims enjoy the claimed priority date.
Therefore, document D4 has not been considered to be part of the prior art as defined in the regulations (Rule 64(1)-(3) PCT).

3. Re Item V
Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

3.1. Claims 1-12 are directed to a method of removing part of a transgene after its integration into a genome by intrachromosomal homologous recombination in the attachment P region (attP) comprising seq. ID no 1, whereby the use of the attP region yields a high frequency of intrachromosomal homologous recombination.
D1 describes a system wherein a gene of interest and a resistance gene are integrated into a host by recombination using modules of site specific recombination of Tn1545 and phage λ. The resistance gene is flanked by λattR and λattL sites and can be excised after integration into the host genome by a

helper phage which bears the necessary phage proteins λxis and λint (p.232, Fig.1 and p.236, § 3.3).
Since the recombinant λattL and λattR sites comprise parts of the attP region but nevertheless differ from the attP sequence of claim 1 and since no other prior art teaches a method with the technical features of claim 1, the subject-matter of claim 1 and dependent claims 2-11 is novel over the prior art (Art. 33(2) PCT).
Consequently, claims 12, 13 and 15-21 directed to plants or plant cells produced by the method of claim 1 or containing a transgene flanked by said specific attP region and related methods are also novel (Art. 33(2) PCT).

3.2. Claim 14 is directed to a plant or plant cell comprising recombinant attP regions without further specification of the sequence of said regions.
Since the plant cells obtained in D1 contain recombinant sites that do actually comprise parts of the attP region, the teaching of D1 is novelty destroying to the subject-matter of claim 14 (Art. 33(2) PCT).

3.3. Claim 1 differs from D1 in that the fragment to excise by intrachromosomal homologous recombination is flanked by specific attP sequences comprising seq. ID no 1 of bacteriophage λ, whereas in D1 the fragment (i.e. the drug resistance gene) is flanked by λattL and λattR sites.
The method claimed appears to be an alternative method to the method of D1. However, neither D1 nor any other document of the prior art suggests the use of said specific attP region. Moreover, the applicants show that the method claimed leads to a more efficient removal of the transgene due to a higher recombination frequency between said attP regions and without the need of helper sequences. Hence, the method of claim 1 could not be derived in an obvious manner from the prior art and represents a technical improvement over already known methods.
Consequently, the subject-matter of claim 1 and dependent or related claims 2-13 and 15-21 is also inventive (Art. 33(3) PCT).

3.4. The subject-matter of claims 1-21 is industrially applicable in the field of food industry (Art. 33(4) PCT).

4. Re Item VI
Certain documents cited (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 01 07572 A	1 February 2001	21 July 2000	23 July 1999

5. Re Item VII
Certain defects in the international application

5.1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D3 is not mentioned in the description, nor are these documents identified therein.

6. Re Item VIII
Certain observations on the international application

6.1. The term "functionally equivalent fragment thereof" used in claim 7 is vague and unclear and leaves the reader in doubt as to the meaning of the technical feature to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT).

It will be appreciated that the term marker gene is intended to include genes involved in specific biosynthetic pathways and/or genes involved in environmental tolerance.

Preferably, the marker gene is selected from the group consisting of *nptII*, Ble, *dhfr*, *cat*, *aphIV*, SPT, *aacC3*, *aacC4*, *bar*, EPSP, *bxn*, *psbA*, *tfdA*, DHPS, AK, *sul*, *crs1-1* and *tdc*.

Preferably, the method is capable of deleting in the region of up to 10 kb between each of the two attP regions and more preferably in the region of 7kb.

Preferably, in the instance of removing more than one marker gene and/or vector sequence and/or other foreign ancillary nucleic acid each undesirable part of the transgene to be removed is flanked by att P regions. Thus it will be appreciated that the method of the invention can simultaneously be used to remove more than one undesired part of the genome at the same time.

Preferably, the attP region comprises 352bp located between position 27492 and 27844 of bacteriophage λ.

Preferably the attP region comprises the nucleic acid sequence as set forth in SEQ ID NO:1, or fragment thereof with the same functional equivalent, or nucleic acids which hybridise under stringent conditions to the DNA of SEQ ID NO:1 and function as an attP region, or nucleic acids which differ from the DNA of SEQ ID NO:1 due to the degeneracy of the genetic code and which function as an attP region.

The method of the invention provides a novel strategy to remove undesirable and/or other parts of a transgene after its integration into a plant genome. The method of the invention exploits the hitherto unrecognised potential of the high recombination efficiency of the attachment P region (attP) of bacteriophage λ, producing deletion events after intrachromosomal recombination between two attP regions. The attP system has been demonstrated to delete a 5.9kb region from a recombinant vector

Claims

1. A method of removing a part of a transgene after its integration into a genome comprising flanking said part of the transgene on each side thereof with an attachment P region (attP) of bacteriophage λ, the attP region comprises a nucleic acid sequence as set forth in SEQ ID NO:1 or fragment thereof which maintains the same function, or nucleic acids which hybridise under stringent conditions to the DNA of SEQ ID NO:1 and function as an attP region, or nucleic acids which differ from the DNA of SEQ ID NO:1 due to the degeneracy of the genetic code and which function as an attP region, and inducing a high frequency of intrachromasomal homologous recombination between flanking attP regions whereby said part of the transgene sandwiched therebetween is removed.

2. A method as claimed in Claim 1 characterised in that said transgene comprises a marker gene and/or vector sequence and/or other foreign ancillary nucleic acid.

3. A method as claimed in Claim 1 or Claim 2 characterised in that the marker gene confers resistance to antibiotics and/or herbicide resistance.

4. A method as claimed in any one of the preceding claims characterised in that the marker gene is involved in specific biosynthetic pathways and/or involved in environmental tolerance.

5. A method as claimed in any one of the preceding claims characterised in that the marker gene is selected from the group consisting of *nptII*, Ble, *dhfr*, *cat*, *aphIV*, SPT, *aaaC3*, *aaaC4*, *bar*, EPSP, *bxn*, *psbA*, *tfdA*, DHPS, AK, *sul*, *crs1-1* and *tdc*.

6. A method as claimed in any one of the preceding claims characterised in that more than one marker gene and/or vector sequence and/or foreign nucleic acid part is

removed from the transgene and each such part is to be removed is flanked by an attP region.

7. A method as claimed in any one of the preceding claims characterised in that the attP region comprises 352 basepairs, or functionally equivalent fragment thereof, located between positions 27492 and 27844 of bacteriophage λ.

8. A method as claimed in any one of the preceding claims characterised in that the attP regions are in a cassette.

9. A method as claimed in Claim 8 characterised in that the cassette further includes a transformation booster sequence or fragment thereof for enhancing homologous and illegitimate recombination.

10. A method as claimed in Claim 8 or Claim 9 characterised in that the cassette includes an effector gene such as oryzacyctastin-I or functional equivalent thereof.

11. A method as claimed in any one of the preceding claims characterised in that the genome is a plant genome.

12. A plant or plant cell or plant tissue whenever produced by the method of any one of Claims 1 to 11.

13. A method which comprises performing the method of Claim 11 to produce a plant or providing a plant or plant cell or plant tissue of Claim 12 and, in either case growing the plant and/or harvesting products therefrom.

14. A plant or plant cell or plant tissue comprising recombinant attP regions.

15. An attP recombination cassette comprising a marker gene and/or vector sequence and/or foreign ancillary nucleic acid flanked on either side by an attP region

AMENDED SHEET

the attP region comprising a nucleic acid sequence as set forth in SEQ ID NO:1 or fragment thereof which maintains the same function, or nucleic acids which hybridise under stringent conditions to the DNA of SEQ ID NO:1 and function as an attP region, or nucleic acids which differ from the DNA of SEQ ID NO:1 due to the degeneracy of the genetic code and which function as an attP region.

16. Use of an attP recombination cassette of Claim 15 for removing a part integrated into a plant genome.

17. A kit for removing a part of a transgene after its integration into a plant genome comprising an attP recombination cassette as claimed in Claim 15.

18. A plant or plant cell or plant tissue comprising a recombinant transgene integrated into its genome characterised in that the transgene is associated with a bacteriophage λ attP region on respective sides thereof, the attP region comprising a nucleic acid sequence as set forth in SEQ ID NO:1 or fragment thereof which maintains the same function, or nucleic acids which hybridise under stringent conditions to the DNA of SEQ ID NO:1 and function as an attP region, or nucleic acids which differ from the DNA of SEQ ID NO:1 due to the degeneracy of the genetic code and which function as an attP region.

19. A plant or plant cell or plant tissue as claimed in Claim 18 characterised in that it includes one such bacteriophage λ attP region and one effector transgene integrated into its genome.

20. A plant or plant cell or plant tissue as claimed in Claim 19 characterised in that the bacteriophage λ attP regions and one transgene are not associated with a marker gene and/or vector sequence and/or other foreign ancillary nucleic acid.

21. A plant or plant cell or plant tissue as claimed in any one of Claims 18 to 20 characterised in that the transgene is further associated with a transformation booster

AMENDED SHEET

sequence or fragment thereof which is capable of enhancing homologous and illegitimate recombination.

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